京都大学	博士（薬科学）	氏 名	廖　文卿
論文題目	Development of Nanostructured DNA Aimed at Enhancing the Stability and Antigen-presenting Cell Targetability of CpG Oligodeoxynucleotide (CpG オリゴデオキシヌクレオチドの安定性および抗原提示細胞指向化の増強を目指したナノ構造化 DNA の開発)		

（論文内容の要旨）

Unmethylated cytosine-phosphate-guanine oligodeoxynucleotide (CpG ODN), which binds to Toll-like receptor 9 (TLR9) and activates antigen-presenting cells (APCs), has been studied as one of the nucleic acid drugs to stimulate the innate immunity. Recent advances on DNA nanotechnology have greatly opened the potential applications of DNA assemblies for CpG ODN delivery and largely solved two major issues upon the application of CpG ODN: degradation by nuclease and delivery to TLR9. However, most DNA nanostructures developed so far have complex structures, like DNA dendrimer, so sequences need to be properly designed. On the other hand, lack of the cellular recognition/targeting property largely limits the further *in vivo* application because its uptake by immune cells just relies on the concentration and stereochemical structures. These problems lead to a high synthetic cost in practical application of CpG ODN. To solve these problems, therefore, the applicant developed simple strategies (1) to deliver the CpG ODN by nanostructuration using G-quadruplex just introducing several guanines into the sequence of CpG ODN and (2) to modify the DNA nanostructures including polypod-like structured nucleic acid and G-quadruplex structured CpG ODN by mannose to increase the delivery efficiency of CpG ODN to APCs.

Chapter 1　Development of G-quadruplex Structured CpG ODN for Enhanced Stability and Immunoreactivity

G-quadruplex (GQ) structure, a stable and naturally existing helical secondary nucleic acid structure, is formed by stacking of G-quartets, guanine tetrads tightened by Hoogsteen interaction with coordinating metal cations (e.g. Na^+, K^+). The superior stability of GQ structure shows its potential in application to nucleic acid drug delivery. In this study, the applicant designed a CpG ODN with only one G-tract (5 guanines) to construct a G-quadruplex structured CpG ODN with precise structural properties and activity to induce potent immune responses. Phosphodiester CpG ODN with the same sequence as CpG1668 was selected and G-tract was added to the 5′-end [1668(5′-G_5)], 3′-end [1668(3′-G_5)] or internally within the sequence [1668(mid-G_5)]. Circular dichroism analysis showed that all the CpG ODN with a G-tract formed parallel GQ structures irrespective of the location of the G-tract. In addition, electrophoresis showed GQ structured 1668(5′-G_5) formed dimer, whereas others remained monomeric GQ structures. Single-stranded or GQ structured CpG ODN was added to TLR9-positive mouse macrophage-like RAW264.7 cells, and tumor necrosis factor (TNF)-α and interleukin (IL)-6 release from the cells was measured. GQ structured CpG ODNs induced greater secretion of both TNF-α and IL-6 than single-stranded CpG ODNs, with the highest secretion from 1668(3′-G_5). Cellular uptake study showed significantly increased uptake of CpG ODN after GQ structure formation. In addition, 1668(3′-G_5) showed a slow decomposition in serum than 1668(5′-G_5). These results indicated that the G-quadruplex formed by only one G-tract was a simple and efficient strategy for the delivery of CpG ODN to immune cells, and that addition of G-tract to 3′-end was effective in obtaining monomeric GQ structured CpG ODN with high biological stability and high immunostimulatory activity.

Chapter 2　Development of Mannose-modified Nanostructured DNA for Targeted Delivery of CpG ODN to Antigen-presenting Cells

In our laboratory, the polypod-like structured nucleic acid (polypodna), a DNA nanostructure constructed with three or more ODNs, has been developed as a novel delivery system for delivery of CpG ODN and apparent enhancement in antitumor effects. However, the lack of cellular recognition property largely limits its further *in vivo* application. Mannose receptor (MR), primarily expressed on the surface of APCs, mediated endocytosis has been studied to achieve cell-specific drug delivery. Therefore, the applicant selected a hexapodna, a polypodna with six pods, and designed a mannose-modified hexapodna to efficiently deliver CpG ODN to the APCs. The modification strategy was to load the mannose-modified CpG1668 (Man-CpG1668) onto the CG sequence-free

hexapodna. Firstly, a mannose motif with a 5 carbons linker, which aimed at increasing its interaction with receptor, was synthesized by 4 steps. Then, CpG1668 with phosphodiester backbone was successfully modified by the mannose motif at 5′-end to give mannose-modified CpG1668 (Man-CpG1668). Man-CpG1668 loaded hexapodna (Man-CpG1668/Hexapodna) was prepared by annealing all ODNs in one-pot and was obtained with high yield. The melting temperature analysis showed mannose modification hardly changed the thermal stability. Man-CpG1668/hexapodna induced greater TNF-α release from TLR9- and MR-positive mouse peritoneal macrophages than CpG1668/hexapodna. These results indicate that the mannose-modified hexapodna is suitable for the delivery of CpG ODN to TLR9-positive immune cells.

Chapter 3 Development of Mannose-modified G-quadruplex Structured CpG ODN for Targeted Delivery to Antigen-presenting Cells

In Chapter 1, a simple strategy to enhance not only the stability but the immunostimulatory activity and internalization efficiency of CpG ODN was successfully achieved by G-quadruplex structuration. In Chapter 2, the immunoreactivity of CpG1668/hexapodna was further enhanced by mannose modification. Based on these results, in Chapter 3, to further expand the capability of GQ structured CpG ODN, the applicant attempted to construct a mannose-modified GQ structured CpG ODN. Initially, 1668(5′-G_5) and 1668(3′-G_5) were successfully modified at 3′-end [1668(5′-G_5, 3′-Man)] and 5′-end [1668(5′-Man, 3′-G_5)] separately by the mannose motif which was synthesized in Chapter 2. However, these two modified sequences failed to form the GQ structure due to the steric hindrance and repulsion from the long hydrophobic linker. Therefore, 1668(5′-G_5) and 1668(3′-G_5) were directly modified by α-D-mannopyranosylphenyl isothiocyanate and they could be successfully converted to GQ structures. After mannose modification, 1668(5′-G_5, 3′-Man) induced higher secretion of TNF-α from mouse peritoneal macrophages than unmodified and mannose modified 1668(5′-Man, 3′-G_5). These results suggested that the immunostimulatory activity of GQ structured CpG ODN could be further increased by mannose modification and indicated the higher density of mannose motif might be necessary to increase the interaction of CpG ODN with APCs due to 2-fold of mannose in 1668(5′-G_5, 3′-Man) than 1668(5′-Man, 3′-G_5).

As described above, the applicant clarified that the stability, delivery and immunostimulatory activity of CpG ODN can be improved by introduction of G-quadruplex structure instead of using other nanocarriers. In addition, mannose modification is suitable for enhancement of delivery of CpG ODN loaded nanostructures. The results in this study not only reveal the potential in simplification of delivery system for CpG ODN, but also provide useful information for antigen-presenting cell-targeted strategy in practical application of CpG ODN.

（論文審査の結果の要旨）

Unmethylated cytosine-phosphate-guanine oligodeoxynucleotide (CpG ODN), which binds to Toll-like receptor 9 (TLR9) and activates antigen-presenting cells (APCs), has been studied as one of the nucleic acid drugs to stimulate the innate immunity. Recent advances on DNA nanotechnology have greatly opened the potential applications of DNA assemblies for CpG ODN delivery and largely solved two major issues upon the application of CpG ODN: degradation by nuclease and delivery to TLR9. The applicant developed simple strategies (1) to deliver the CpG ODN by nanostructuration using G-quadruplex just introducing several guanines into the sequence of CpG ODN and (2) to modify the DNA nanostructures including polypod-like structured nucleic acid and G-quadruplex structured CpG ODN by mannose to increase the delivery efficiency of CpG ODN to APCs.

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The applicant designed a CpG ODN with only one G-tract (5 guanines) to construct a G-quadruplex (GQ) structured CpG ODN with precise structural properties and activity to induce potent immune responses. Phosphodiester CpG ODN with the same sequence as CpG1668 was selected and G-tract was added to the 5′-end [1668(5′-G_5)], 3′-end [1668(3′-G_5)] or internally within the sequence [1668(mid-G_5)]. Circular dichroism analysis showed that all the CpG ODN with a G-tract formed parallel GQ structures irrespective of the location of the G-tract. In addition, electrophoresis showed GQ structured 1668(5′-G_5) formed dimer, whereas others remained monomeric GQ structures. Single-stranded or GQ structured CpG ODN was added to TLR9-positive mouse macrophage-like RAW264.7 cells, and tumor necrosis factor (TNF)-α and interleukin (IL)-6 release from the cells was measured. GQ structured CpG ODNs induced greater secretion of both TNF-α and IL-6 than single-stranded CpG ODNs, with the highest secretion from 1668(3′-G_5). Cellular uptake study showed significantly increased uptake of CpG ODN after GQ structure formation. In addition, 1668(3′-G_5) showed a slow decomposition in serum than 1668(5′-G_5). These results indicated that the G-quadruplex formed by only one G-tract was a simple and efficient strategy for the delivery of CpG ODN to immune cells, and that addition of G-tract to 3′-end was effective in obtaining monomeric GQ structured CpG ODN with high biological stability and high immunostimulatory activity.

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以上、本研究で得られた知見は、CpG ODN のデリバリー研究に対して有用な基礎的情報を提供するものと考えられる。よって、本論文は博士（薬科学）の学位論文として価値あるものと認める。また、令和 2 年 2 月 14 日、論文内容とそれに関連した事項について試問を行った結果、合格と認めた。